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Differential expression of arachidonate 5-lipoxygenase transcripts in human brain tumors: Evidence for the expression of a multitranscript family

(leukotrienes/cell biology/neoplasia/gene regulation/RNA)

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ABSTRACT **In addition to the important role of leukotrienes as mediators in allergy and inflammation, these compounds are also linked to pathophysiological events in the brain including cerebral ischemia, brain edema, and increased permeability of the blood–brain barrier in brain tumors. Although brain tumors have been shown to secrete leukotrienes, no studies to date have provided evidence for the tumor expression of genes encoding enzymes involved in leukotriene production. Therefore, the present study determined the abundance of the mRNA for arachidonate 5-lipoxygenase (5-LO; arachidonate:oxygen 5-oxidoreductase, EC 1.13.11.34), which is the rate-limiting enzyme in leukotriene synthesis, in a series of human brain tumors. Macrophage/monocyte infiltration of the tumor was estimated by measuring the abundance of the transcript for the 91-kDa glycoprotein phagocyte-specific oxidase (gp91-phox), which is the phagocyte-specific cytochrome *b* heavy chain. The present study shows that (*i*) the 5-LO transcript is expressed in normal bovine brain and in human brain tumors; (*ii*) the 5-LO gene in human brain tumors and in the dimethyl sulfoxide-induced promyelocytic human leukemic HL-60 cells is expressed as a multitranscript family (2.7, 3.1, 4.8, 6.4, 8.6 kilobases); and (*iii*) the abundance of 5-LO transcripts, the expression of the larger transcripts, and the 5-LO/gp91-phox ratio correlate with the tumor malignancy. Overall, the present study supports the hypothesis that the 5-LO gene product may play a role in human tumor-induced brain edemas and provides evidence for tumor-associated expression of high molecular weight 5-LO transcripts in human brain tumors.**

Leukotrienes, important mediators of allergy and inflammation, are synthesized from arachidonic acid by 5-lipoxygenation to 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5-HPETE) and to leukotriene A_4 (1–3). The limiting step in this reaction is catalyzed by the enzyme arachidonate 5-lipoxygenase (5-LO; arachidonate:oxygen 5-oxidoreductase, EC 1.13.11.34), whose gene was recently cloned from human lung and HL-60 cDNA libraries (4, 5). The expression of the 5-LO transcript was correlated with the cellular synthesis of leukotrienes (6). Although the product of the 5-LO gene is mainly found in leukocytes, lung, and placenta (1, 4), it has been postulated that metabolites of 5-LO may be involved in the brain with respect to modulation of the release of neurotransmitters (7, 8). This is supported by the findings that immunoreactive leukotriene C_4 is present in the nerve endings in the median eminence and in cell bodies in the preoptic area (9). Although indirect evidence for leukotriene production in neuronal tissue was obtained in experimental animals (9, 10) and humans (11), no direct determination of the 5-LO gene product has yet been performed for the human or experimental animal brain. Therefore, one of the purposes of the present study was to measure the abundance of the 5-LO transcript in bovine and human brain.

Leukotrienes have also been linked to pathophysiological events in the brain. The concentration of leukotrienes in brain is increased in cerebral ischemia (12). Leukotrienes also play a role in brain edema (13, 14). Moreover, administration of leukotriene C_4 selectively increases the permeability of the blood–brain barrier in brain tumors (15), suggesting that these molecules may mediate the vasogenic edema associated with brain tumors. Cerebral meningiomas and astrocytomas are hypothesized to release cysteinylleukotrienes to blood at rates that correlate with the grade of malignancy (16). If this were the case, then the level of the 5-LO mRNA in brain tumors may also correlate with malignancy.

The analysis of 5-LO mRNA levels in human brain tumors is complicated by the invasion of the tumor by blood-derived phagocytic cells, which may also express 5-LO mRNA. Although human brain tumors are immunosuppressive (17), these tumors often show evidence of infiltration by macrophages/monocytes. Therefore, the present study also evaluated the abundance of the mRNA for the 91-kDa glycoprotein phagocyte-specific oxidase (gp91-phox), which is the heavy subunit of phagocyte-specific cytochrome b and is transcribed in terminally differentiated phagocytes (18).

MATERIALS AND METHODS

Materials. Tetra(triethylammonium) salt of deoxycytidine 5′-[α-^{32}P]triphosphate (dCTP) (3000 Ci/mmol; 1 Ci = 37 GBq), Cronex Lightning Plus intensifying screens, and GeneScreen*Plus* membranes were purchased from DuPont/NEN. Megaprime DNA labeling system was obtained from Amersham. Restriction endonucleases were purchased from United States Biochemical. Ultrapure-DNA-grade agarose was obtained from Bio-Rad. All other molecular-biology-grade reagents were obtained from Sigma.

The human 5-LO cDNA in the plasmid pUC13 (5) was kindly provided by Ron Diehl (Merck Sharp & Dohme Research Laboratories). Double restriction-enzyme digestion of the plasmid with *Eco*RI and *Pvu* I, followed by agarose gel electrophoresis and ethidium bromide staining, resulted in the release of the 2.8-kilobase (kb) cDNA insert and smaller (1.9 and 1.1 kb) fragments of the pUC13 plasmid. The mouse actin cDNA clone pAM-91 (19) was provided by Michael J. Getz (Mayo Foundation, Rochester, MN). A human cy-

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Abbreviations: 5-, 12-, and 15-LO, arachidonate 5-, 12-, and 15-lipoxygenases; gp91-phox, 91-kDa glycoprotein phagocyte-specific oxidase.

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tochrome *b* heavy chain cDNA probe (gp91-phox) (18), corresponding to the 1600-base-pair (bp) *Pst* I/*Sac* I fragment and subcloned in Bluescript KS, was provided by Mary Dinauer (Indiana University Medical Center, Indianapolis).

Human intracranial neoplastic tissues were obtained from patients who underwent craniotomy for therapeutic reasons. Immediately after resection, the tissue was placed in cryovials, deep-frozen in liquid N_2, and kept at −70°C before isolation of poly(A)$^+$ RNA. Bovine brain tissue was obtained fresh from a local slaughter house. The human promyelocytic leukemic HL-60 cell line (ATCC CCL 240) obtained from the American Type Culture Collection was grown in Iscove's modified Dulbecco's medium containing 20% (vol/vol) fetal bovine serum (HyClone). HL-60 cells were differentiated by the addition of 1.2% dimethyl sulfoxide to the incubation medium 5 days before the isolation of cell RNA (6).

Poly(A)$^+$ RNA Isolation and Northern Blotting Analysis. Bovine brain, human brain tumors, and HL-60 poly(A)$^+$ RNAs were isolated by using a single-step method described recently (20). Briefly, 1-g aliquots of tissue or 10^8 dimethyl sulfoxide-treated HL-60 cells were homogenized in 25 ml of lysis buffer (10 mM Tris, pH 7.5/1 mM EDTA/0.2 M NaCl/2% sodium dodecyl sulfate/400 μg of proteinase K per ml) for 5 sec in a Polytron PT 3000 homogenizer (Brinkmann) at 12,000 rpm. After digestion of cellular proteins for 45 min at 45°C, poly(A)$^+$ RNA was bound to oligo(dT)-cellulose in 0.5 M NaCl lysis buffer for 30 min at room temperature. The resin was washed twice with 25 ml of 10 mM Tris, pH 7.5/1 mM EDTA/0.5 M NaCl, followed by an additional wash of 25 ml of the same buffer containing 0.1 M NaCl instead of 0.5 M NaCl. Poly(A)$^+$ RNA was then eluted in 10 mM Tris, pH 7.5/1 mM EDTA by using a spin X filter unit (Costar) (20). The yield of human brain tumor poly(A)$^+$ RNA averaged 23.5 ± 2.8 μg/g of tissue (mean ± SE, n = 11), and the A_{260}/A_{280} ratio was 2.65 ± 0.03, reflecting the degree of RNA purity. Poly(A)$^+$ mRNA (10 μg per lane for brain tumors and 2–8 μg per lane for HL-60 cells) was applied to 1.1% agarose/2.2 M formaldehyde gels, followed by blotting onto GeneScreen*Plus* membranes. Hybridization with [^{32}P]cDNA for 5-LO, gp91-phox, or actin was performed under conditions (50% formamide, 42°C) as described (21, 22). After autoradiography with intensifying screens, Northern blots were quantified by scanning optical densitometry of the x-ray film with the MicroScan 1000 two-dimensional gel analysis system (Technology Resources, Nashville, TN). Plasmids encoding 5-LO and actin were digested with *Eco*RI, whereas that encoding gp91-phox was linearized with *Pst* I, and were labeled with [α^{32}P]dCTP to a specific activity of ≈10^9 cpm/μg by the multiprimer technique (23).

RESULTS

Northern blot analysis for 5-LO transcripts in poly(A)$^+$ RNA from human brain tumors, bovine brain, and HL-60 cells is shown in Figs. 1 and 2. The human 5-LO [^{32}P]cDNA probe hybridized with a single band of ≈2.8 kb in relatively low abundance in bovine brain (Fig. 1 *Left*, lane 1). In dimethyl sulfoxide-differentiated human HL-60 cells, a series of multiple 5-LO transcripts ranging from 2.7 to 8.6 kb were observed (Fig. 1 *Left*, lane 2). The relative abundance of the various 5-LO mRNAs in HL-60 cells calculated by integration of the areas determined by scanning densitometry of the autoradiogram shown in Fig. 2 is 42.9%, 30.4%, 21.9%, 3.2%, and 1.6% for the 2.7-, 3.1-, 4.8-, 6.4-, and 8.6-kb bands, respectively.

The levels of 5-LO mRNA in human tumors were studied in two different experiments. In the first experiment (Fig. 1 *Right*), the most abundant 5-LO transcripts (2.7, 3.1, and 4.8 kb) were detected in two meningiomas (lanes 1 and 2) and in a spinal cord ependymoma (lane 3). Hybridization with actin showed comparable 2.1-kb bands corresponding to the cytoplasmic β and γ isoforms (24). In the second experiment, similar results were obtained in another meningioma (Fig. 2,

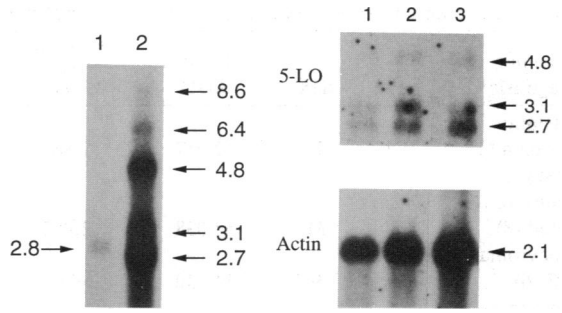

FIG. 1. (*Left*) Northern blot with 5-LO cDNA probe of 10 and 8 μg of poly(A)$^+$ mRNA isolated from bovine brain (lane 1) and dimethyl sulfoxide-activated HL-60 cells (lane 2), respectively. The membrane was hybridized with the 5-LO [^{32}P]cDNA and exposed for 3 days at −70°C. A single 2.8-kb transcript is found in bovine brain (lane 1), whereas a 5-LO multitranscript family (2.7, 3.1, 4.8, 6.4, and 8.6 kb) is observed in the HL-60 cells (lane 2). (*Right*) Northern blot with 5-LO (*Upper*) and actin (*Lower*) cDNA probes of poly(A)$^+$ mRNA isolated from two meningiomas (lanes 1 and 2) and a spinal cord ependymoma (lane 3). Ten micrograms of poly(A)$^+$ RNA of each sample was applied and, after hybridization with the 5-LO [^{32}P]cDNA probe, autoradiography was performed for 16 hr at −70°C. The membrane was unhybridized and rehybridized with actin [^{32}P]cDNA, and the film was exposed 4 days at room temperature. The sizes of the transcripts are indicated in kb and were estimated by comparing the migration distance with that of a parallel RNA ladder detected by ethidium bromide staining.

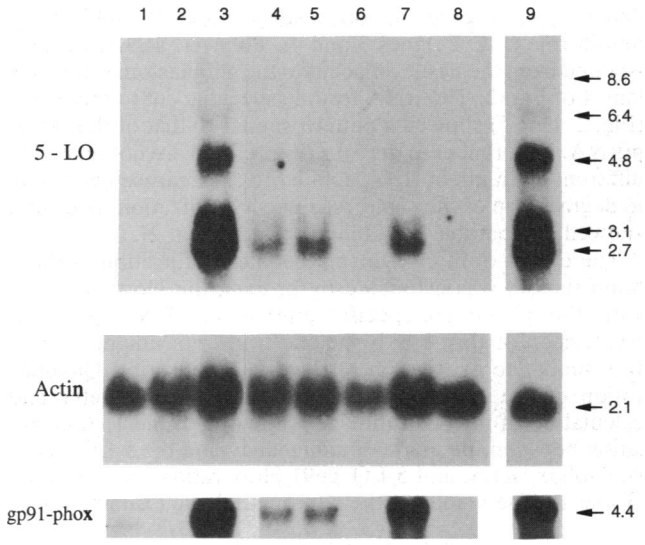

FIG. 2. Northern blot with 5-LO, actin, and gp91-phox cDNA probes of poly(A)$^+$ mRNA isolated from human brain tumors and dimethyl sulfoxide-treated HL-60 cells. Lanes: 1, anaplastic glioma, grade III; 2, metastatic poorly differentiated sarcoma; 3, glioblastoma multiforme; 4, glioblastoma multiforme; 5, brain tissue adjacent to the glioblastoma applied in lane 4; 6, astrocytoma grade I–II; 7, meningioma; 8, metastatic undifferentiated carcinoma of unknown origin; 9, dimethyl sulfoxide-differentiated HL-60 cells. Ten micrograms of poly(A)$^+$ mRNA of brain tumors and 2 μg of HL-60 cells were applied. Membrane was subsequently hybridized with [^{32}P]cDNAs for 5-LO, actin, and gp91-phox, and the exposure time and temperature with each probe were respectively 16 hr at −70°C, 16 hr at 22°C, and 3 days at −70°C. The size of the hybridization bands is indicated in kb. The 8.6- and 6.4-kb bands were faintly visible on the x-ray film for both lanes 3 and 9. The 2.7-kb band was faintly visible on the x-ray film for lanes 1, 2, and 8.

Table 1. Differential expression of 5-LO, actin, and gp91-phox (gp91) mRNAs in human brain tumors

Tumor diagnosis*	Age	Sex	Actin area, ADU	5-LO area, ADU		5-LO/actin ratio		gp91 area, ADU	gp91/actin ratio	5-LO/gp91 ratio	
				2.7 kb	3.1 kb	2.7 kb	3.1 kb			2.7 kb	3.1 kb
Metastatic sarcoma (2)	51	F	52,957	2,861	ND	0.054	0	ND	0	—	—
Metastatic undifferentiated (8)	24	M	52,038	2,082	ND	0.040	0	2,136	0.041	0.97	—
Astrocytoma I–II (6)	35	F	34,330	ND	ND	0	0	ND	0	—	—
Astrocytoma III (1)	43	F	43,168	1,794	ND	0.042	0	4,444	0.103	0.40	0
Glioblastoma IV (4)	78	F	43,888	12,103	5,710	0.276	0.130	11,717	0.267	1.03	0.49
Glioblastoma BAT (5)	78	F	46,065	15,966	6,218	0.347	0.135	12,897	0.280	1.24	0.48
Glioblastoma IV (3)	52	M	67,384	108,849	47,114	1.615	0.699	59,128	0.877	1.84	0.80
Meningioma (7)	68	M	59,530	18,491	7,641	0.310	0.128	39,952	0.671	0.46	0.19
HL-60 (9)	—	—	37,153	64,206	44,258	1.728	1.19	43,597	1.173	1.47	1.02

Autoradiograms shown in Fig. 2 were quantified by optical scanning densitometry, and the area obtained was expressed in arbitrary densitometric units (ADU) and summarized in this table. ND, not detectable; BAT, brain adjacent tumor.
*Number in parentheses represents the lane number in Fig. 2. Roman numerals indicate the grade of malignancy.

lane 7). The 5-LO transcripts were barely detected in brain tumors of metastatic origin—i.e., sarcoma (Fig. 2, lane 2) or primary tumor of unknown origin (Fig. 2, lane 8); see also Table 1. There was an association between 5-LO transcripts and the grade of malignancy. The 5-LO mRNA was not detected in the astrocytoma grade I–II (Fig. 2, lane 6), and a faint 2.7-kb band was seen on the x-ray film for a grade III anaplastic glioma (Table 1). Conversely, high levels of 5-LO transcripts, including 6.4- and 8.6-kb species of low abundance, were observed in both cases of grade IV glioblastoma multiforme (Fig. 2, lanes 3 and 4). Poly(A)$^+$ RNA was also isolated from the tissue adjacent to the glioblastoma shown in lane 4 of Fig. 2. The RNA from tissue adjacent to the tumor (Fig. 2, lane 5) showed a pattern similar to that of the tumor mRNA. Differences in the 5-LO expression are not related to different amounts of RNA applied to the agarose gel or due to degradation of the samples, since hybridization with actin showed comparable 2.1-kb actin bands (Fig. 2).

The extent of monocyte/macrophage infiltration in these brain tumors was estimated by probing the Northern blots with the phagocyte-specific gp91-phox cDNA (Fig. 2). Expression of this 4.4-kb specific transcript among tumors was similar to expression of the 5-LO transcript. Quantification of the results by laser scanning densitometry and calculation of mRNA ratios are shown in Table 1. A correlation between the grade of malignancy and the 5-LO/actin, gp91-phox/actin, and 5-LO/gp91-phox ratios was observed (Table 1). The results of the neuropathologic examination of the histology of each of the 10 human brain tumors studied in Figs. 1 and 2 are shown in Table 2 with respect to a qualitative evaluation of the tumor infiltration by blood-borne phagocytic cells.

DISCUSSION

The present study demonstrates that transcriptional products of the 5-LO gene are expressed in control bovine brain and in human brain tumors. This investigation also shows, in addition to the already reported 2.7-kb 5-LO transcripts (4, 5), that the 5-LO gene is expressed as a multitranscript family (2.7, 3.1, 4.8, 6.4, and 8.6 kb) in a variety of malignant and benign brain tumors and in the differentiated promyelocytic leukemic HL-60 cells.

The 5-LO transcript in bovine brain is a single band of 2.8 kb (Fig. 1 *Right*), and previous studies also report predominant hybridization of the 5-LO probe to a single 2.7-kb species in human lung, placenta, and leukocytes (4). Although a single 2.7-kb transcript was also reported in dimethyl sulfoxide-differentiated HL-60 cells, these studies were performed with 10 μg of total RNA (5). However, 2–8 μg of HL-60 poly(A)$^+$ mRNA was used in the present study, and consequently the 5-LO transcripts other than 2.7-kb species were detected (Figs. 1 and 2). We considered that the 5-LO cDNA probe cross-hybridized with other transcripts of the lipoxygenase gene superfamily. However, an analysis of the nucleotide similarity using the data base of GenBank and the

Table 2. Neuropathologic examination of tumor histology

Tumor	Identification	Acute hemorrhage	Necrosis	Inflammation	
				Acute	Chronic
Ependymoma	Fig. 1, lane 3	−	−	−	++
Meningioma	Fig. 1, lane 2	−	−	−	++
Meningioma	Fig. 1, lane 1	+	−	−	−
Meningioma	Fig. 2, lane 7	−	−	−	−
Metastatic	Fig. 2, lane 2	++	+++	+	Trace
Metastatic	Fig. 2, lane 8	+++	−	−	−
Grades 1–2	Fig. 2, lane 6	−	−	−	−
Grade 3	Fig. 2, lane 1	++	−	−	−
Grade 4	Fig. 2, lane 3	−	++	+	++
Grade 4	Fig. 2, lane 4	−	++	−	−

−, None; +, mild; ++, moderate; +++, marked.

FASTA program (25) showed that the 5-LO nucleotide sequence is only 55.9% and 55.5% identical to the human arachidonate 12- and 15-lipoxygenase (12-LO and 15-LO), respectively (26, 27). Therefore, under the high-stringency hybridization conditions used in the present study, it is unlikely that there is cross-hybridization of the 5-LO cDNA probe with 12- and 15-LO transcripts. Moreover, the 12-LO gene transcript was reported to be a single species of 2.4 kb (28). The molecular cloning of the 5-LO gene showed that there is a major transcriptional initiation site in human leukocytes at the thymidine residue localized 65 bp upstream from the methionine initiation codon, and this accounts for the transcription of the 2.7-kb species (29). Potential minor initiation sites were also localized within the 107 nucleotides of the ATG 5′ flanking region (29), suggesting that one of these putative initiation sites may be responsible for the expression of the second most abundant transcript (3.1 kb; Figs. 1 *Left* and 2). Since neither TATA nor CCAAT boxes were found within 250 bp of the transcription initiation sites (29), it is possible that the expression of the largest 5-LO transcripts (4.8, 6.4, and 8.6 kb, respectively; Figs. 1 and 2) were products of as-yet-unidentified multiple promoters located further upstream in the 5′ flanking region of the 5-LO gene. However, these 5-LO gene products may also be related to aberrant splicing of the primary 5-LO transcript in tumor cells.

The 5-LO transcripts were barely detectable in the metastatic tumors of human brain (Fig. 2), and there is an association between the abundance of these mRNAs and the grade of malignancy of primary brain tumors that we studied. For example, the 5-LO gene product was not expressed in grades I–II glioma, was barely expressed in grade III glioma, but was highly expressed in the grade IV gliomas. One of the grade IV gliomas (Fig. 2, lane 3) even showed the least abundant 6.4- and 8.6-kb transcripts, which were only detected in the dimethyl sulfoxide-differentiated leukemic HL-60 cells. The benign meningiomas also showed expression of the three most abundant 5-LO transcripts (2.7, 3.1, and 4.8 kb). However, when the densitometry results are normalized by using the phagocyte-specific gp91-phox cDNA, the 2.7- and 3.1-kb 5-LO/gp91-phox ratios are 2.2- to 4.2-fold higher in glioblastomas than in meningiomas (Table 1).

The level of 5-LO mRNA in human brain tumors was normalized with the measurement of the mRNA for gp91-phox (18), which is assumed to be specific for phagocytic macrophages/monocytes that invade the tumor from blood. However, this phagocytic-specific mRNA may also be expressed in human primary brain tumors. For example, the meningioma that expresses the gp91-phox mRNA (Fig. 2, lane 7) exhibited no histologic signs of necrosis or acute or chronic inflammation (Table 2). Conversely, the poorly differentiated sarcoma metastatic to brain expressed no detectable gp91-phox mRNA (Table 1) but showed histologic evidence of extensive necrosis, acute inflammation, and mild chronic inflammation (Table 2). Therefore, we hypothesize that the 5-LO mRNA transcripts of varying size expressed in glioblastoma multiforme and to a lesser extent in meningiomas arise from the tumor cells *per se* and not from infiltrating phagocytic blood cells. The gp91-phox gene may also be differentially expressed in the brain tumors.

In summary, the present studies provide direct evidence for 5-LO gene expression in bovine (Fig. 1 *Left*) and human (Figs. 1 *Right* and 2) brain. Although these studies are based on a relatively small sample size (Table 2), the presence of a 5-LO multitranscript family is demonstrated for both meningiomas and glioblastoma multiforme (Figs. 1 and 2). Both of these tumors are the primary brain tumors that exhibit breakdown of the blood–brain barrier (30). Therefore, these results are consistent with the hypothesis that tumor-derived leukotriene production contributes to the increased permeability of the blood–brain barrier in certain brain tumors (13, 15, 31).

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